Understanding the risks of co-exposures in a changing world: A case study of dual monitoring of the biotoxin domoic acid and Vibrio spp. in Pacific oyster

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Abstract

Assessing the co-occurrence of health risk factors in coastal ecosystems is challenging due to limited co-located environmental monitoring data. Understanding co-occurrence is particularly important for risk factors that may be associated with or occur in similar environmental conditions, such as harmful algal bloom toxins and bacterial pathogens within the genus *Vibrio*. This study examined the co-occurrence of *Vibrio* spp. and domoic acid (DA) by analyzing existing California Department of Public Health monitoring data of *V. parahaemolyticus* and DA, and conducting seasonal monitoring for these risk factors across two Southern California embayments. Existing public health monitoring efforts in the state were robust for individual risk factors, however it was difficult to evaluate the co-occurrence of these risk factors in oysters collected around the same date (5-day window) between 2015 and 2020. Co-monitoring of two Southern California embayments revealed that DA and *Vibrio* spp. (*V. vulnificus* or *V. parahaemolyticus*) co-occurred in 34% of sampled oysters in most seasons. Interestingly, both the overall detection frequency and co-occurrence of these risk factors was considerably less frequent in water samples. These findings expand our understanding of the simultaneous presence of DA and *Vibrio* spp. in bivalves and demonstrate the feasibility of co-monitoring different risk factors from the same sample. Individual programs monitoring for different risk factors from the same sample matrix may consider combining efforts to reduce cost and streamline the process and better understand prevalence of co-occurring health risk factors.

Introduction

Water quality can become compromised by a variety of chemical and biological factors that impact human and/or ecological health. These factors can originate from both human activities and natural sources, with environmental conditions influencing their presence and persistence. Temperature increases from climate change and anthropogenic eutrophication, for example, are both elements that encourage harmful algal bloom formation, as well as promote growth of pathogenic bacteria, including certain strains of *Vibrio* spp. (Rosales et al. 2022). As our understanding of these risks expands, the need to monitor and conduct risk assessments for diverse environmental and human health factors also increases.

Relatively few studies and monitoring programs examine the co-occurrence of multiple risk factors. While independent monitoring of individual factors simplifies analysis and results interpretation, it hinders a comprehensive understanding of the interactions between different risk factors and the effects of a changing environment on them. Predictive water quality, hydrodynamic, and biogeochemical models often require information on risk factors and environmental conditions collected in tandem. An improved understanding of the relationships between risk factors and the corresponding environmental parameters that promote them may ultimately enable the identification and prioritization of high-risk sites, along with the streamlined implementation of effective mitigation strategies. However, untangling these complex relationships necessitates the collection and utilization of more comprehensive datasets.

Segregated and focused funding is one major reason for independent monitoring of different types of risk factors. However, the total cost for monitoring risk factors individually increases significantly due to repeated field sampling effort, often duplicating staff time and resources for the collection of samples as well as ancillary data. Under the current paradigm of siloed monitoring programs, government and monitoring agencies invest substantial resources in routine water quality monitoring that generates a large amount of independent data that does not always allow for integrated analyses.

Using paired sampling of *Vibrio* spp. and domoic acid (DA; an algal toxin) occurrence in oyster and water samples from two southern California embayments as an example, we present a case study where the seasonal co-occurrence of *V. parahaemolyticus, V. vulnificus*, and DA within the same sample matrices over a one-year period is investigated. A subset of public health monitoring data for *V. parahaemolyticus* and DA in California between 2015 and 2020 were also evaluated. *V. parahaemolyticus* and *V. vulnificus* are both potentially pathogenic bacteria that are the most common cause of human seafood-related gastrointestinal infection or mortality, respectively. Both of these *Vibrio* species occur naturally in marine and brackish waters and have been found to thrive in warm and eutrophic conditions (Canellas et al. 2021; Rosales et al. 2022). Such conditions are also conducive to the formation of harmful algal blooms (HABs), some of which are formed by species that can produce potent toxins. *Pseudo-nitzschia* is a genus of marine diatom with some species that can produce the neurotoxin DA. Shifts in nutrient regimes and increases in temperature have been shown to increase growth and toxin production in harmful algal species, including certain species of *Pseudo-nitzschia* (Lema et al. 2017; Thorel et al. 2014). Hence, climate change and eutrophication are elements that may enhance the growth of pathogenic *Vibrio* spp. and toxic harmful algal bloom species in the environment, which may be further concentrated in filter feeding organisms such as oysters (Fig. 1a). As oysters are commonly harvested for human consumption, the presence of algal toxins and pathogenic bacteria in oysters and other filter feeding organisms may pose a high risk to human health. Such risks may be exacerbated by increasing anthropogenic and climate driven change.

Materials and methods

Public health monitoring of *V. parahaemolyticus* and DA in oysters between 2015–2020

The presence of *V. parahaemolyticus* and DA are routinely monitored in shellfish for the protection of public health by the Shellfish Program run by the Environmental Management branch of the California Department of Public Health (CDPH). CDPH ensures that commercial shellfish conforms to the standards of the National Shellfish Sanitation Program (NSSP). The risk for DA in commercial shellfish growing areas is evaluated weekly by testing shellfish samples (High Performance Liquid Chromatography method, limit of detection = 2.5 µg/g) and/or evaluating *Pseudo-nitzschia* abundances in water samples. The Shellfish Program also monitors for *V. parahaemolyticus* in oyster meat samples (PCR, limit of detection = 3.0 MPN/g) from commercial growing areas, ideally bi-weekly during months identified as higher risk for *V. parahaemolyticus* illness.

Data from oyster samples analyzed for *V. parahaemolyticus* and DA from 2015–2020 were examined for co-occurrence of these factors (Fig. 1b). While CDPH has an extensive dataset on DA risk from a variety of shellfish including mussels, oysters, and clams, only data from oysters were considered here to match the data available for *V. parahaemolyticus*. The data considered for each factor are from distinct monitoring efforts and were analyzed at separate labs. We focused our evaluation on data from four embayments located in four counties (Fig. 1c; DA = 619 samples, *V. parahaemolyticus* = 146 samples). When there were multiple sites per embayment, individual sampling sites were binned to one general embayment. All oysters sampled were cultured Pacific oysters (*Crassostrea gigas)*, with the exception of 2 samples for DA analysis that were Kumamoto Oyster (*Crassostrea sikamea)* from Humboldt Bay.

Study design and sample processing for *Vibrio* spp. and domoic acid co-occurrence study

Water and oyster samples from two embayments in Southern California (Fig. 2a), Newport Bay (NPB; two stations; Fig. 2b) and San Diego Bay (SDB; three stations, Fig. 2c), were collected to investigate the co-occurrence of *V. parahaemolyticus, V. vulnificus*, and DA. Samples were collected in four seasons (Fall: Sep – Oct 2020, Winter: Jan – Mar 2021, Spring: Apr – May 2021, and Summer: Jun – Jul 2021), with three sampling events per season. Water temperature was measured by a Hydrolab HL4 sonde (OTT HydroMet, Loveland, CO, USA), and 4 L of water (collected in two acid-washed 2 L polycarbonate bottles and kept in a cooler with ice packs until further processing) were collected at the time of oyster collection.

Ten Pacific oysters (*C. gigas*) from existing natural beds were collected at each station during each sampling event, placed in a cooler with icepacks, and transferred to the laboratory where they were processed immediately. The oyster shells were shucked and the oyster tissues were kept frozen until they were homogenized individually using a handheld tissue homogenizer (Omni, Kennesaw GA, USA). The homogenate was then split for *Vibrio* spp. and DA analyses (Fig. 2d).

Water collected during sampling was processed in the laboratory for *Vibrio* spp., particulate domoic acid (pDA), and chlorophyll *a* (chl-*a*) concentration analyses. Water was filtered through a 25 mm GF/F (0.7 µm; Steriltech, Auburn, WA, USA) filter for chl-*a* (100 mL) and pDA (200–500 mL) analysis and stored at -20^{o}C until processing. *Vibrio* spp. samples were collected by filtering 100 mL of water through a 47 mm 0.45 µm polycarbonate filter (Cytiva, Marlborough, MA, USA) and stored at -80^{o}C until processing.

Chl-*a* samples were extracted with 90% acetone for 24 hours at -80^{o}C, then measured on a Trilogy Fluorometer (Turner Designs, San Jose, CA, USA) using a non-acidification module. DA samples (from both water and oyster) were extracted with aqueous methanol (10% for pDA samples; 50% for oyster DA samples) and measured using an enzyme linked immunosorbent assay (ELISA) test kit (Mercury Science, Durham, NC, USA) following manufacturer's instructions. Samples for pDA from SDB in summer 2021 were lost. Filters for *Vibrio* spp. detection were extracted using Zymobiomics Magbead extraction kit (Zymo, Irvine, CA, USA), following the manufacturer's instructions for the KingFisher Flex system (Thermo Scientific, Waltham, MA, USA). Measured aliquots (5–20 mg) of oyster homogenates were placed in DNA/RNA shield (Zymo, Irvine, CA, USA), and extracted using the Zymobiomics Magbead extraction kit

(Zymo, Irvine, CA, USA) following the manufacturer's instructions. Three homogenized oyster samples were used for DA and *Vibrio spp.* analyses per sampling event for each sampling site.

V. vulnificus hemolysin (VVHA) gene was amplified using VVHA-F (3'-TGTTTATGGTGAGAACGGTGACA-5'), VVHA-R (5'-TTCTTTATCTAGGCCCCAAACTTG-3') primers, and probe VVHA-P (3'-CCGTTAACCGAACCACCCGCAA-5'). *V. parahaemolyticus* toxT transcriptional activation gene (TOXR) was amplified using TOXR-F (3'-GAACCAGAAGCGCCAGTAGT-5'), TOXR-R (5'-AAACAAGCAGTACGCAAATCG-3') primers, and probe TOXR-P (3'-TCACAGCAGAAGCCACAGGTGC-5'). The probes were dual-labeled with a 5' 6-carboxyfluorescein FAM fluorescent tag and a 3' Black Hole Quencher. Absolute concentrations of amplified genes were quantified using BioRad QX200 Droplet Digital PCR (ddPCR) system (BioRad, Hercules, CA, USA). ddPCR was performed following the procedure in Cao et al. (2016), with quality control parameters utilized as described in Steele et al. 2018. The following cycling protocol was used: hold 95°C 10 min, 40 cycles of 94°C 30s and 60°C 1 min, hold 98°C for 10 min. Each plate contained at least two no-template control (NTC; RNA/DNA-free water; UltraPureTM, Life Technologies, Carlsbad, CA, USA) reactions and two positive control (genomic DNA extracted from *Vibrio spp.*) reactions. The plate was then analyzed on the QX200 Droplet Reader using QX Manager software (v.1.2, BioRad). Copies per volume water or per gram tissue were calculated in R (R Core Team, 2021, https://github.com/kylielanglois/SCCWRP/tree/main/ddPCR).

A total of 178 oyster samples measured for *Vibrio* genes and 162 samples measured for DA were included in further analysis, with a total of 160 oyster samples measured for both DA and *Vibrio* genes. Non-parametric Mann-Whitney rank sum and Kruskal-Wallis tests were used to test the difference in DA and *Vibrio* genes between pairs and groups of samples, respectively. Kendall's rank correlation tests were used to assess statistical associations between parameters. These statistical tests were performed using the base packages in R (version 4.2.2).

Results and discussion

Public Health Shellfish Data collected from four CA locations between 2015–2020

While CDPH tests routinely for both DA and *V. parahaemolyticus* in commercial oyster samples across the four embayments considered, the samples were rarely collected on or near the same date between 2015–2020 (Fig. 1b). Out of a total of 619 DA and 146 *V. parahaemolyticus* data analyzed, there were 26 instances where oysters from the same embayment were tested for both risk factors on the same day. Each of these co-monitoring instances occurred at Morro Bay in San Luis Obispo County, CA, where the majority of DA samples were collected (579 of 619 samples). Adding a lag/lead time of 2 days (i.e. a window of 5 days: sampling day and +/- 2 days) increased the number of co-monitoring instances to 38 across all embayments, with the exception of Humboldt Bay. No co-occurrence of DA and *V. parahaemolyticus* was found in oysters collected on/around the same date at the same site. Notably, co-monitoring for these factors is not currently a NSSP standard, thus these programs were not specifically designed to assess co-occurrence due to variable sample collection designs (e.g., weekly monitoring

across the year for DA vs. bi-weekly monitoring during high-risk months). Thus, despite the extensive monitoring efforts of CDPH for individual parameters, these design differences made it difficult to estimate the prevalence of DA and *V. parahaemolyticus* co-occurrence from existing monitoring data.

While numerous studies have investigated HABs, algal toxins, and *Vibrio* spp. independently in shellfish, we did not find any studies that examined both risk factors from the same shellfish tissues. Studies that did measure both HABs/algal toxins and *Vibrio* spp. were limited (Asplund et al. 2011; Greenfield et al. 2017; Rosales et al. 2022). However, despite the limited scope, these studies consistently indicated that increased *Vibrio* spp. abundance and HAB occurrence (and likely also algal toxins) are influenced by similar environmental factors, including warmer temperatures, expansion of brackish environments because of rising sea levels, and eutrophication (Fig. 1a). In addition, studies have shown that *Vibrio* spp. abundance can be associated with various algal blooms (Asplund et al. 2011; Diner et al. 2022), and that certain phytoplankton species may augment the growth of *Vibrio* spp. (Greenfield et al. 2017).

Paired Oyster and Water Samples Collected from two CA Embayments in 2020–2021

Despite similar environmental drivers, synchronized monitoring of algal toxins and *Vibrio* spp. is limited. Therefore, we conducted a case study that investigated the presence of DA, *V. parahaemolyticus*, and *V. vulnificus* in oysters collected from two embayments in Southern California. *V. parahaemolyticus*, *V. vulnificus*, and DA have all been detected previously in shellfish from the region (Diner et al. 2022; Smith et al. 2018). Furthermore, as climate change continues to lead to increased temperature in more northern areas along the Pacific coast, these embayments in Southern California may serve as valuable sentinels of future conditions.

Water samples had low and mostly undetectable levels of DA and *Vibrio* spp. genes (TOXR and VVHA). pDA was only detected in spring at SDB2 (2 of 51 pDA samples; Fig. 2e), while > 70% of water samples had undetectable copies of *Vibrio* spp. genes (Fig. 2h). VVHA was only detectable in water samples from SDB1 during winter and spring (3 of 60 samples). TOXR was detectable in both NPB and SDB in all seasons except spring in NPB (16 of 60 samples). While *Vibrio* spp. are often associated with warmer water conditions, the water temperatures of the studied embayments in Southern California were > 13^{o}C even during winter, which is generally above the lower temperature preference of the studied *Vibrio* spp. (10–15^{o}C; Brumfield et al. 2021; Kaspar and Tamplin 1993).

Unlike in water samples, DA and *V. vulnificus* genes (VVHA) were commonly detected in oyster samples. Over 70% of oyster samples (119 of 162 samples) had detectable DA, and DA was detected in oysters from both embayments and during all four seasons captured (Fig. 2f). The mean DA concentration in all oysters was 41 ng/g (n = 119), about 500x lower than the U.S. FDA shellfish safety level (< 20,000 ng/g; U.S. Food and Drug Administration 2021). The highest concentrations of DA were measured in spring (82 ng/g; $p < 0.05$). This seasonality coincides with the typical *Psuedo-nitzschia* bloom and DA peak season in this region (Smith et al. 2018). More than 40% (74 of 178 samples) of oyster samples had detectable VVHA genes, with the mean number of copies for summer samples (7,790 copies/g) significantly higher

than the mean for winter (2,795 copies/g) and spring samples (1,326 copies/g; $p < 0.01$; Fig. 2i). Interestingly, VVHA was less frequently detectable in water samples but more frequently detected in oyster tissues when compared to TOXR (Fig. 2h & i).

The two studied embayments, located within relatively close (< 200 km) proximity, exhibited similar temperature chl-*a* concentrations (Fig. 2g & j). There was no significant difference in temperature and chl-*a* concentrations between the two regions (NPB and SDB; $p > 0.05$). However, there was a significant difference in concentration of TOXR and VVHA genes, with higher counts in NPB compared to SDB ($p < 0.05$). Number of *Vibrio* genes and concentration of DA detected in water were generally not correlated with that detected in oyster tissues ($p > 0.05$) except for TOXR in the summer ($p < 0.05$, $\tau = 0.50$).

V. parahaemolyticus, and *V. vulnificus* genes in water and oyster tissues had some correlations with temperature and chl-*a* concentrations, while DA in both water and oyster tissues had no significant correlation with temperature or chl-*a*. TOXR water column levels were positively correlated with chl-*a* concentrations ($p < 0.005$, $\tau = 0.31$), while VVHA water column concentrations were negatively correlated with temperature ($p < 0.05$, $\tau = -0.23$). In contrast, there was positive correlation between VVHA concentration in oyster tissues and temperature in NPB ($p < 0.001$, $\tau = 0.54$). The negative correlation between temperature and the number of VVHA genes in water was surprising given *V. vulnificus'* association with warmer temperatures. However, while it is often documented that *V. vulnificus* growth was inhibited by low temperatures, water temperatures throughout this study remained within the growth range of *V. vulnificus* (Kaspar and Tamplin 1993), thus other factors, besides temperature may be contributing to the levels observed in the water column and oyster tissues.

In accordance with the high frequency of DA and *V. vulnificus* genes detected in oyster tissues in this study, DA and the two *Vibrio* spp. co-occurred in > 30% of oyster samples. Forty-one of 160 oyster samples measured for both DA and *Vibrio* genes had detectable DA and VVHA, while an additional 10 oyster samples had detectable DA and TOXR. Furthermore, 4 oyster samples had detectable DA, VVHA, and TOXR. Co-occurrence of DA and at least one of the two measured *Vibrio spp.* occurred in both embayments in most seasons (except in spring at SDB). However, there was no significant correlation between *Vibrio* genes in water and DA in water, and a slight negative correlation between VVHA in oysters and DA in oysters ($p < 0.01$, $\tau = -0.16$). Nevertheless, while simultaneous detection of all three risk factors (DA and two *Vibrio* spp.) measured in this study was rare (< 3% of 160 oyster samples) and there was no significant positive correlation between *V. parahaemolyticus* or *V. vulnificus* and DA in oysters, our results indicated that these risk factors indeed co-occur and can be measured from the same sample.

The co-occurrence of multiple health risk factors in various environments is attracting increasing attention within the scientific community (e.g. Khoshmanesh et al. 2023; Metcalf and Codd 2020). Filter-feeders like oysters, in particular, can increase exposure as their feeding mode may concentrate both pathogens and toxins. Furthermore, oysters are often consumed raw, increasing the health risk to humans. Adequate cooking is considered effective in eliminating the risk of *Vibrio* infection (Brumfield et al. 2021), and the harvest of DA and *Vibrio* contaminated shellfish are reduced based on evaluation by

public health monitoring programs by annual quarantines and harvesting advisories. Nevertheless, multiple types of risk factors may still be present in one matrix that may have synergistic harmful effects on both environmental and human health. For example, Pacific oysters exposed to the harmful algal species *Alaxandrium catenella* were more susceptible to *Vibrio tasmaniensis* infections that cause mass mortalities in the oysters themselves (Abi-Khalil et al. 2016). DA is also not the only algal toxin of concern in coastal shellfish, as freshwater cyanobacterial toxins like microcystins have been detected in coastal and estuarine waters and shellfish in multiple locations within California (Howard et al. 2023). Chatterjee and More (2023) suggested a potential link between human patients suffering from liver diseases caused by the freshwater cyanobacterial hepatotoxin microcystin and their increased susceptibility to severe *Vibrio* infections (Baker-Austin and Oliver 2018). Furthermore, chronic exposure to below regulatory limit of algal toxins may lead to chronic conditions and illness. Chronic exposure to DA has been suggested to have neurotoxic effects on humans such as decreased memory performance (Petroff et al. 2021). It is unknown how additional exposure to pathogenic *Vibrio* species may affect patients with underlying health conditions.

Conclusions

The results of our study confirmed the presence of both an algal toxin (DA) and potential pathogenic *Vibrio* spp. (*V. parahaemolyticus* and *V. vulnificus*) in paired oyster samples collected from two embayments in Southern California. While the levels of DA detected in oysters were well below the FDA shellfish safety level, and the risk of *Vibrio* can be eliminated by proper hygiene procedures (e.g., adequate cooking), the main goal of the study was to demonstrate the possibility of monitoring multiple health risk factors from the same matrices. This study used the marine algal toxin DA and *Vibrio* spp. as examples for co-monitoring, but there are many other contaminants (e.g., heavy metals) that should be considered depending on the location of interest. The implications of co-occurring health risk factors and the potential for synergistic effects on human and organismal health are still being explored. This is particularly important in to understand for oysters and other shellfish that are commercially and recreationally harvested for human consumption. Future work should examine the co-occurrence of other types of risk factors. Site specific risk assessments are a path forward to identify and prioritize monitoring of potential co-occurring risk factors. Individual programs monitoring different risk factors from the same matrix may then consider joining forces as demonstrated in this study, reducing the resources and effort required to monitor for multiple risk factors.

Declarations

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Figures

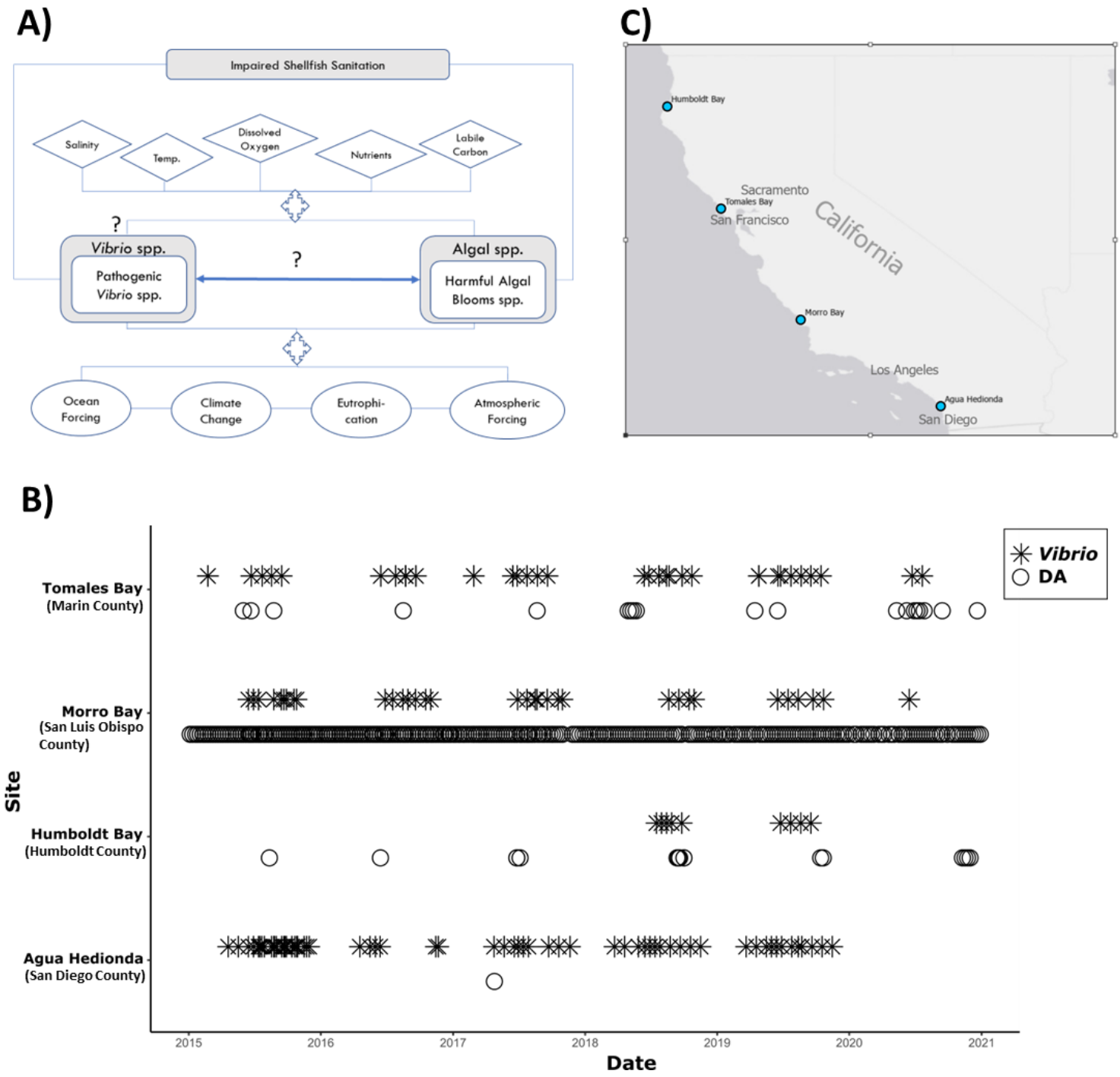

Figure 1

A) Conceptual model of *Vibrio* spp. abundance, potential covariates, and their associations evaluated in this study. B) Monitoring effort of *V. parahaemolyticus* and domoic acid (DA) in oysters at 4 different sites in California by the California Department of Public Health from 2015 - 2020. C) Map of the 4 sites in California that are included in the CDPH dataset presented in B).

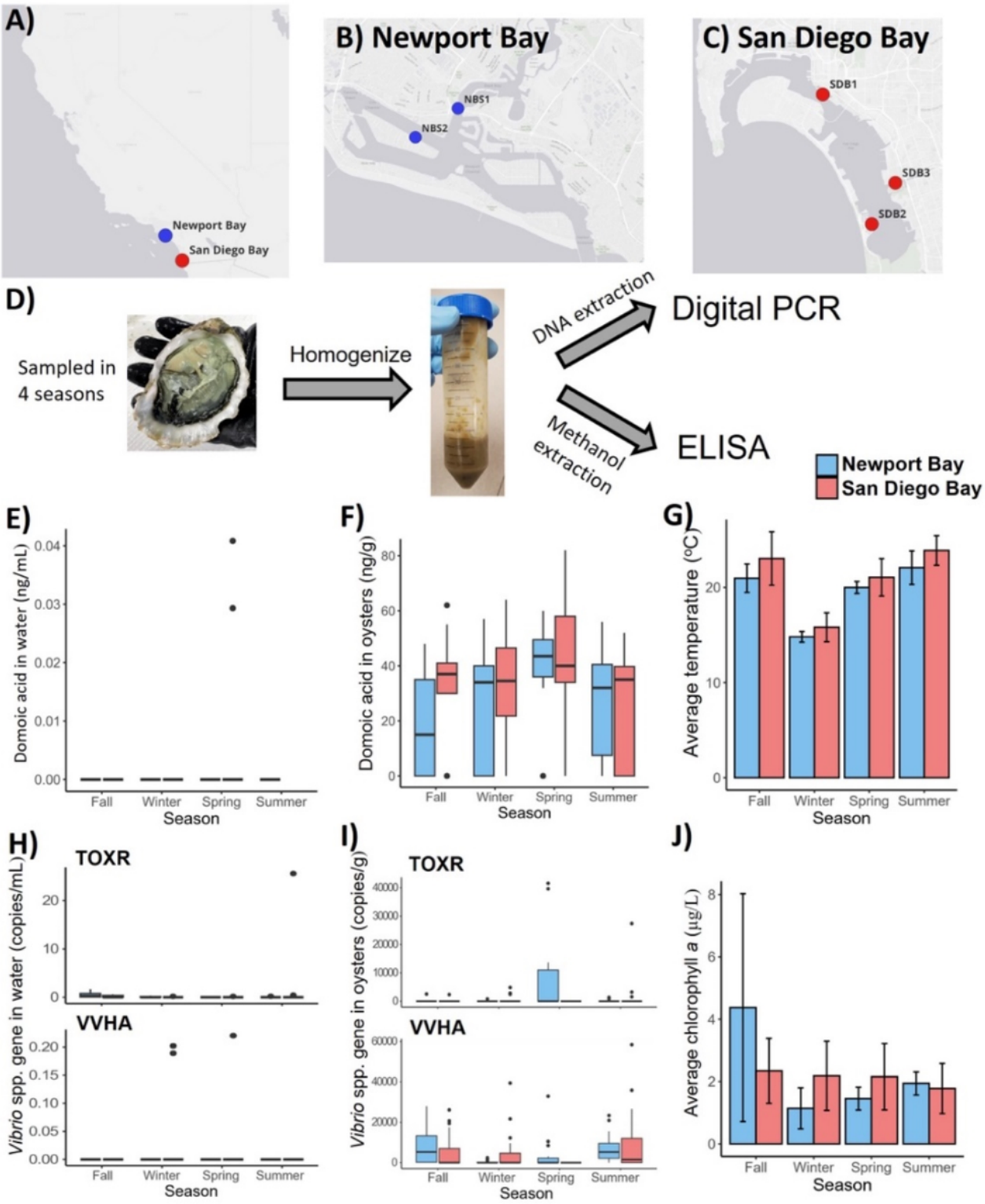

Figure 2

A) Map of California showing the two studied embayments in Southern California. B) Map of Newport Bay (NPB) with the two sampling sites. C) Map of San Diego Bay (SDB) with the three sampling sites. D) Overview of the oyster sample processing in this study. E) Box plots of the concentrations of particulate domoic acid (DA) from water samples collected along oyster samples. Data for SDB in Summer is unavailable. F) Box plots of the concentrations of DA from oyster samples. G) Average temperature (error

bars = standard deviation; *n*=6 in NPB, *n*=9 in SDB) during sample collection. H) Box plots of copy numbers of *Vibrio parahaemolyticus* toxT transcriptional activation gene (TOXR) and *Vibrio vulnificus* hemolysin gene (VVHA) in water samples. I) Box plots of copy numbers of TOXR and VVHA in oyster samples. J) Average chlorophyll *a* concentrations (error bars = Standard deviation; *n*=6 in NPB, *n*=9 in SDB) in water samples.